Exhibit B

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Review

Differential response to ischemia in adjacent hippocampal sectors: neuronal death in CA1 *versus* neurogenesis in dentate gyrus

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Two hippocampal sectors show distinct responses to transient ischemia: the cornu Ammonis (CA)1 sector undergoes a delayed neuronal death followed by a lack of neuronal generation, while the dentate gyrus (DG) shows slight postischemic damage followed by an increased neurogenesis. Using the monkey experimental paradigm of transient whole brain global ischemia, the 'calpain-cathepsin hypothesis' was formulated in 1998. This hypothesis proposes that following ischemia calpain compromises the integrity of lysosomal membrane, causing a leakage of degrading hydrolytic enzymes – cathepsins – into the cytoplasm. Ischemia induces Ca^{2+} mobilization, calpain activation, lysosomal membrane disruption, and cathepsin release, which all occur specifically in the CA1 sector and cause neuronal death. In the postischemic DG, a vascular niche has been implicated in adult neurogenesis, in that adventitial cells of the DG microvascular environment provoke postischemic up-reguation of neurogenesis with the aid of brain-derived neurotrophic factor and polysialylated form of the neural cell adhesion molecule. In parallel, Down's syndrome cell adhesion molecule has recently been shown to be expressed specifically in the neural progenitor cells of DG. In this review, we focus on the monkey experimental paradigm to reveal the remarkable contrasts between CA1 and DG in response to the ischemic insult.

Keywords: Cerebral ischemia · Down's syndrome · Hippocampus · Neurogenesis · Neuronal death

1 Background

From rodents [1, 2] to primates [3–6], transient brain ischemia is well known to cause delayed neuronal death in the hippocampal cornu Ammonis (CA)1 sector. Delayed neuronal death is complete within 5–7 days after the ischemic insult, but it still remains uncertain which cascade, apoptosis or necrosis, is mainly involved in CA1 neuronal death. Whether ischemic neuronal death occurs by apoptosis or necrosis, depends not only on the experimental paradigm "global or focal" and "transient or permanent ischemia", but also on the experimental subjects "rodents or primates" and "fetal or adult brain" [7]. Cysteine proteases, such as caspases, have been claimed to be a key factor for the apoptosis cascade especially in rodents [8], because caspase inhibitors can attenuate certain types of neuronal death [9–12]. Mechanisms other than caspase inhibition, however, have been shown to exert neuroprotective effects in the primates; cathepsin inhibitor CA-074 and E64c do not block caspases but nonetheless inhibit neuronal necrosis of the ischemic monkeys. Accordingly, Yamashima *et al.* [13] formulated

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Abbreviations: BDNF, brain-derived neurotrophic factor; **BrdU**, bromodeoxyuridine; **CA**, cornu Ammonis; **DG**, dentate gyrus; **DSCAM**, Down's syndrome cell adhesion molecule; **GCL**, granule cell layer; **LAMP-2**, lysosome-associated membrane protein-2; **PSA-NCAM**, polysialylated neural cell adhesion molecule; **ROS**, reactive oxygen species; **SGZ**, subgranular zone

the 'calpain-cathepsin hypothesis' in 1998 that posits a critical role of other types of cysteine proteases, calpain (EC 3.4.22.17) and cathepsins B (EC 3.4.22.1) and L (EC 3.4.22.15), on executing neuronal necrosis [7, 13]. Subsequently, calpain and cathepsin have been confirmed to play an essential role in neurodegeneration in *Caenorhabditis elegans* [14]. With accumulating evidence implicating the calpain-cathepsin cascade in species from *C. elegans* to primates, the roles of these two cysteine proteases have been confirmed in many experimental paradigms and animals as well as in human degenerative diseases [15–32].

Neurogenesis is the process of neuronal generation by brain stem/progenitor cells. In mammals including rodents [33–37] and primates [38–40], it has been established that new neurons are generated in the dentate gyrus (DG) of the adult hippocampus. Neuronal progenitor cells (immature neuronal precursors) proliferate in the subgranular zone (SGZ) of the DG: the thin lamina of approximately 100 μm between the hippocampal hilus (CA4) and the granule cell layer (GCL). Young neurons in the SGZ gradually develop morphological and functional properties of dentate granule cells and become integrated into pre-existing neuronal circuitries of the GCL [41]. In both rodent [42, 43] and primate hippocampi [44–47], neuronal production is known to be up-regulated after an ischemic insult. The recruitment of neural precursor is considered to be crucial for the neurogenesis. Palmer *et al.* [48] reported that, in the rat SGZ, neurogenesis occurs in a vascular microenvironment via endothelial precursors. Later, at the ultrastructural level, Yamashima *et al.* [46] found in the postischemic monkey SGZ that the vascular adventitia is a potential source of neuronal progenitor cells. Dore-Duffy *et al.* [49] confirmed this finding by the isolation and culture analyses of rat primary pericytes, showing that microvessels of the central nervous system contain NG2- and nestin-positive pericytes that can replicate in response to basic fibroblast growth factor (bFGF) and differentiate into astrocytes, oligodendrocytes or neurons. Further, Jin *et al.* [50] confirmed in human stroke patients that newly formed neurons in the ischemic penumbra are preferentially localized in the vicinity of nestin-positive adventitial cells.

The monkey experimental paradigm of the transient global ischemia [6] is appropriate for considering the mechanisms of both CA1 neuronal death and adult DG neurogenesis occurring in the same hippocampus. The two events of neuronal death and genesis occur in adjacent brain areas and at comparable time points; neuronal death is complete within the 1st week in the CA1, while neurogenesis becomes maximal at the 2nd week after ischemia in the neighboring DG. This review discusses the mechanisms of ischemic neuronal death and adult neurogenesis, with particular attention being paid to the role of lysosomal disruption and the vascular niche. Here we claim that cathepsins released from lysosomes are the final executers of neuronal necrosis, while ischemia-induced vascular proliferation is indispensable for adult neurogenesis.

2 Anatomy of monkey hippocampus

The Japanese monkey (*Macaca fuscata*) brain is quite similar to the human brain. Although much smaller than the human hippocampus, the basic architecture of monkey hippocampus is similar to humans, being located in the medial temporal lobe of the brain. Compared to the rodents, however, the hippocampus of primates occupies less of the cerebrum in proportion to the cerebral cortex, but is much larger. The anatomist Giulio Cesare Aranzi (circa 1564) first used the term 'hippocampus' to denote its visual resemblance to a 'seahorse'. A coronal section of the hippocampus reveals the internal structure as two interlocking 'Cs' with one being reversed in relation to the other. One C makes up Ammon's horn comprising sectors CA1–4 (frequently called hilus) (Fig. 1a), while the other C makes up the DG. Although the DG is commonly included as a part of the hippocampus, it is cytoarchitectonically distinct from the hippocampus proper [51–54].

Fura-2 imaging *in vitro* of hippocampal acute slices with hypoxia-hypoglycemia using an Argus camera [6]

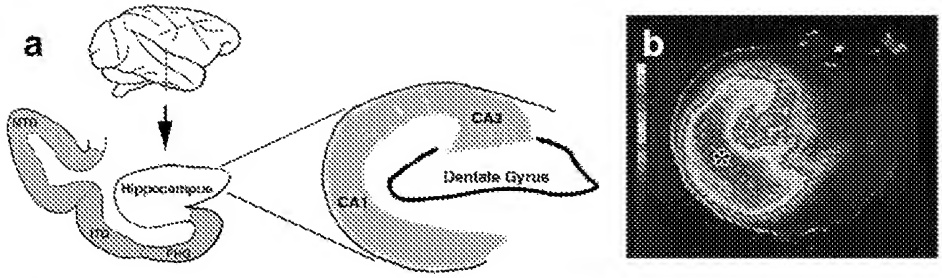

Figure 1. The schematic architecture of monkey hippocampus and Ca^{2+} imaging. (a) A coronal section of the hippocampus reveals two interlocking „Cs"; one C is the CA comprising sectors CA1–4, while the other C is the DG. CA1 shows delayed neuronal death in the 1st week after ischemia, while the DG shows neurogenesis in the postischemic 2nd week. MTG, middle temporal gyrus; ITG, inferior temporal gyrus; PHG, parahippocampal gyrus. (b) Ca^{2+} imaging of the hippocampal acute slice using an Argus camera (Hamamatsu Photonics, Japan) and Fura-2 clearly demarcates the CA1 sector (asterisk), showing the area of the greatest Ca^{2+} mobilization during *in vitro* hypoxia-hypoglycemia, which mimics *in vivo* cerebral ischemia.

can clearly define the area of the CA1 sector because this has the greatest Ca^{2+} mobilization (Fig. 1b; shown by the asterisk). The demarcation of the CA1 sector highly corresponds with an area of greatest Ca^{2+} mobilization. This Ca^{2+} mobilization during the ischemic insult specifically occurs in CA1 and serves as a trigger of the calpain-cathepsin cascade, which is a cause of delayed neuronal death.

3 Lysosomal disruption and cathepsin release

Ca^{2+} mobilization in CA1 induces sustained activation of calcium-activated neutral protease calpain within this sector [6, 55]. After activation calpain is transferred from the cytosol to the lysosomal membrane, causing its disruption, which can be confirmed by electron microscopy [13]. Because the lysosome-associated membrane protein-2 (LAMP-2) is physiologically localized at the lysosomal membrane, a wide-spread translocation of LAMP-2 (Fig. 2) seen using immunohistochemistry indicates lysosomal disruption. Laser confocal analysis reveals very fine granules of LAMP-2 in the perikarya of the control CA1 neurons and the surrounding glial cells (Fig. 2, C). In contrast, from immediately after ischemia (Fig. 2, 3 h) up to day 1 (Fig. 2, d1), the immunoreactivity of lysosomes increases, showing a coarse-granular pattern. Importantly, such increase of LAMP-2 immunoreactivity occurs specifically in the CA1 sector, while the neighboring CA2 sector shows almost no immunoreactivity (Fig. 2, d1, left). On day 3 after ischemia (Fig. 2, d3), nearly the entire perikarya of CA1 neurons and also the proximal axons show a remarkable LAMP-2 immunoreactivity depicted by scattering of lysosomal membrane debris. The LAMP-2 signal becomes intense throughout the perikarya, demonstrating a remarkable contrast to the control (Fig. 2, C). As LAMP-2 was localized at the lysosomal membrane before ischemia, such translocation indicates an impaired integrity of the lysosomal membrane. On day 5 (Fig. 2, d5), the number of immunoreactive CA1 neurons and their immunointensity decrease with occurrence of neuronal death. The dynamic change of LAMP-2 was grossly consistent with that of LAMP-1 [55].

The pattern of cathepsin immunoreactivity before ischemia is characterized by small granules colocalized at the lysosomes in the perikarya (Fig. 3, C), but there is no detectable immunoreactivity within the nucleus. In contrast, on day 1 after ischemia (Fig. 3, d1), the cathepsin immunoreactivity shows coarse granules that are partially transferred into the nucleus. This indicates lysosomal membrane disruption, resulting in leakage of cathepsin enzymes into the cytoplasm and transfer into the nucleus (Fig. 3, d1). Translocation of not only cathepsins B and D (Fig. 3) but also cathepsin L [7, 13] and DNase II [56] was demonstrated in the postischemic CA1 neurons from immediately after ischemia until day 3. An alternative explanation of the cathepsin immunoreactivity within the nucleus, other than lysosomal disruption, might be the failure of lysosomal targeting of the newly synthesized cathepsins (*e.g.*, due to cleavage of cytoskeleton by calpains) and misdirection of them to the nucleus.

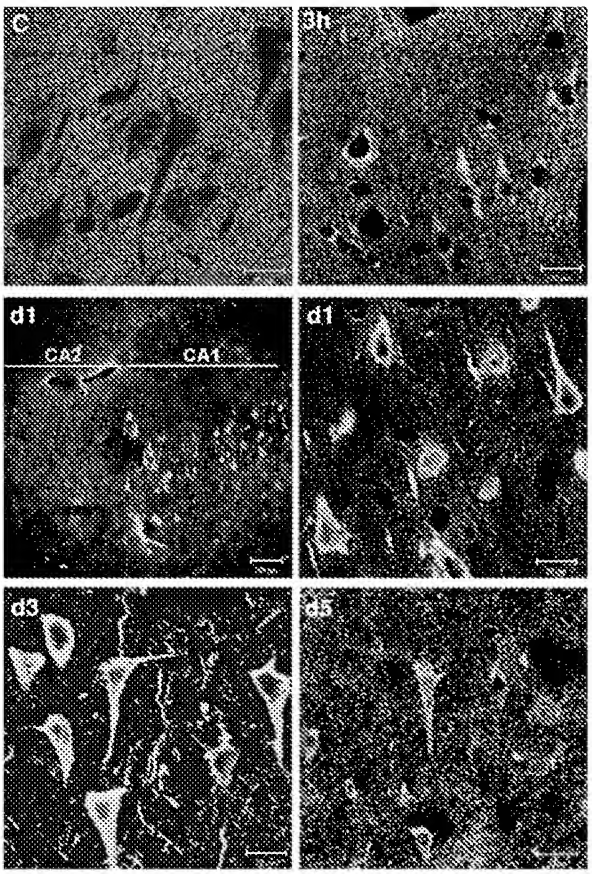

Figure 2. Translocation of LAMP-2 into the perikarya in the CA1 neurons especially on days 1–3 after ischemia. FITC (white) exhibits only punctuated labeling of LAMP-2 in the control CA1 neurons (C). In contrast, from immediately after ischemia (3h) until day 1 (d1) immunoreactivity of lysosomes increases throughout the perikarya especially in CA1 (left, d1). On day 3 after ischemia (d3), LAMP-2 immunoreactivity becomes maximal, then deteriorates until day 5 (d5). Bar, 20 μm (left d1: bar, 100 μm).

4 Morphology of CA1 neurons before and after ischemia

The normal CA1 neurons before ischemia show round and vesicular nuclei by light microscopy (Fig. 4, C). In contrast, on day 7 (Fig. 4, d7), all CA1 neurons reveal eosinophilic coagulation necrosis that is characterized by the reddish, shrunken cytoplasm and diffusely condensed nucleus, but no detectable apoptotic features. Prior to ischemia, the CA1 neuron ultrastructurally displays a vesicular chromatin distribution, an intact cell membrane, and membrane-bound lysosomes (Fig. 4, C). In

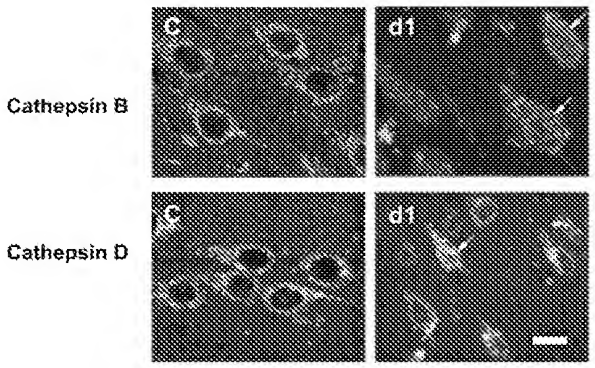

Figure 3. Translocation of cathepsin enzymes after the 20-min whole brain ischemia. The cathepsin B and D immunoreactivities (green) before ischemia (C) are seen as small granules in the perikarya. In contrast, on day 1 after ischemia (d1), they become coarse granules, which are partially transferred into the nuclei (arrows). The nuclei are counterstained red with propidium iodide. Bar, 20 µm.

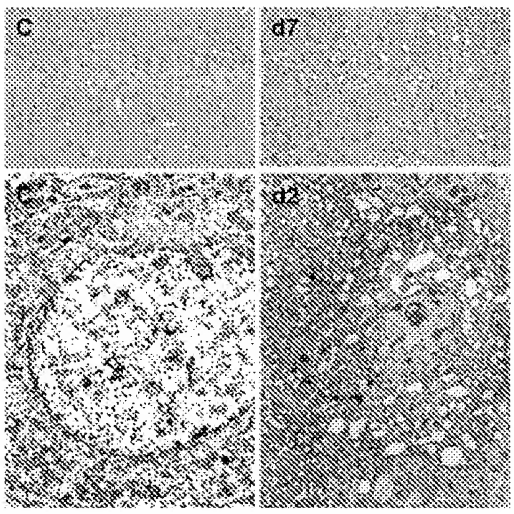

Figure 4. Light and electron microphotographs of the control and postischemic CA1 neurons. Compared to the non-ischemic control (C), the CA1 neurons 7 days after ischemia (d7) disclose eosinophilic coagulation necrosis on hematoxylin-eosin staining (upper). By electron microscopy (lower), the control CA1 neuron (C) shows intact cell membrane and membrane-bound lysosomes. In contrast, the CA1 neuron on day 2 after ischemia (d2) displays marked vacuolation of organella and scattering of the lysosomal granules throughout the cytoplasm (asterisks). Bar, 50 µm (upper), 2 µm (lower).

contrast, the postischemic CA1 neuron on day 2 (Fig. 4, d2) ultrastructurally exhibits punctuated chromatin condensation, but never shows dense chromatin condensation called apoptotic bodies. The cytoplasm becomes more electron dense compared to the control, showing a marked degeneration such as vacuolation of rough endoplasmic reticulum and Golgi apparatus, and swelling of mitochondria. Lysosomal membrane disruption causes scattering of the tiny lysosomal granules throughout the cytoplasm (Fig. 4, d2). The postischemic CA1 neurons on day 5 display obvious membrane disruption and lysis of cell organelles that are characteristic of necrosis (data not shown).

5 Density of vessels and progenitors

Although the CA1 shows an almost total loss of pyramidal neurons up to days 5–7, vessel density is significantly increased in the CA1 compared to the DG on day 4 after ischemia (Fig. 5a). Considering that ELR$^+$ CXC chemokines including IL-8 have potent angiogenic activities, the coordinate expression of ELR$^+$ CXC chemokines and their receptor CXCR2 in the proliferating microglial cells (see below) may be related to this capillary proliferation in the postischemic CA1 [57]. Interestingly, within a few days after the ischemic insult, CA1 shows neuronal loss on the one hand, but vascular proliferation in on the other. As reported in our previous studies using the monkey experimental paradigm [44, 46, 58], the proliferating cells that have incorporated bromodeoxyuridine (BrdU) are observed in both the control and postischemic monkeys. The BrdU$^+$ cell density is significantly more intense in the postischemic hippocampus compared to the control. Dynamic changes of the density of BrdU$^+$ nuclei occur in both CA1 and DG (Fig. 5b). Consistent with the time point of increase of vessel density (Fig. 5a), incorporation of BrdU in the CA1 reaches plateau on day 4, and by day 15 the number of BrdU-labeled cells is more than tenfold that of the control. The increase of BrdU labeling in the DG is less remarkable than that of the CA1. The pattern of postischemic BrdU labeling in the DG is different from that of the CA1, with immunoreactivity reaching peak on day 9 (fivefold tha t of controls) and slightly decreasing by day 15 (fourfold that of controls). These increments are statistically significant as compared to the control.

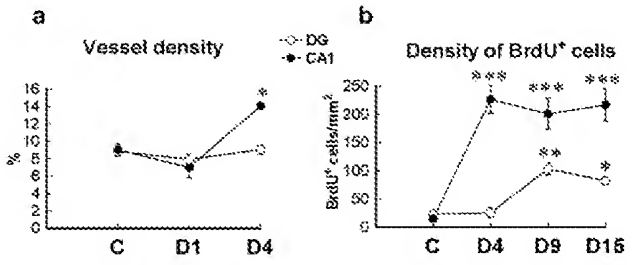

Figure 5. Dynamic change of vessel and progenitor densities. Density of vessels (a) and bromodeoxyuridine (BrdU)$^+$ cells (b) is compared between DG (open circles) and CA1 (closed circles). The pixel numbers of anti-CD31 antibody-positive areas were measured on ten randomly chosen visual fields of each areas at ×100 magnification with the aid of Adobe Photoshop software. BrdU$^+$ nuclei were counted blindly on every 12th section per animal and quantified densitometrically (cells/mm^2). The asterisks indicate *p<0.05, **p<0.01, ***p<0.001, respectively, and the statistical analysis used is one-way ANOVA followed by Fisher's PLSD.

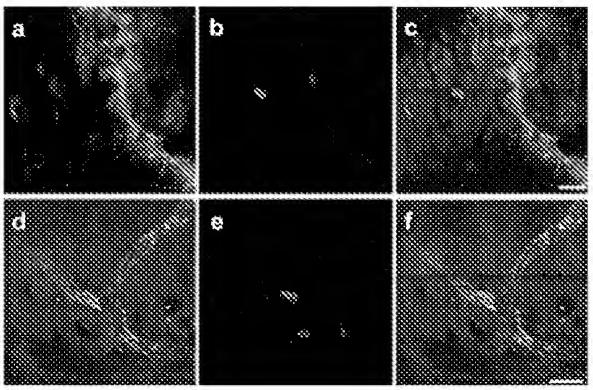

Figure 6. Spatial relationship of the BrdU$^+$ progenitor cells (b, e) to the blood vessels in the DG of day 9 after ischemia. (a–c) One BrdU$^+$ cell is colabeled with a mature neuronal marker NeuN, while another is localized in close proximity with RCA$^+$ vessel. (d–f) One BrdU$^+$ cell is colabeled with an endothelial cell marker von Willebrand Factor, while the other two are not colabeled and localized at the adventitia. Bar, 20 μm.

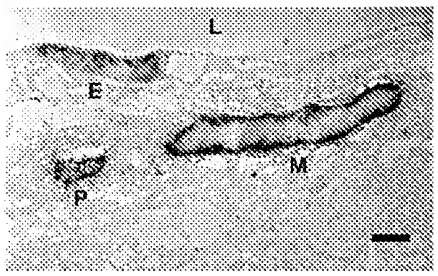

Figure 7. Immunoelectron microscopy of BrdU in the proliferating microvessels of the DG 4 days after ischemia. BrdU$^+$ nuclei are revealed by high electron density, being comprised of endothelial (E) and adventitial cells. The BrdU$^+$ adventitial cells are either Iba1$^+$ microglia (M) or Iba1$^-$ neural progenitor cells (P). No counterstain. L: vascular lumen. Bar, 2 μm.

6 Vascular and microglial proliferation

On day 9, some BrdU$^+$ cells in GCL are colabeled with the mature neuronal marker NeuN, while others are positive for the vascular marker RCA (Fig. 6a–c). To investigate the spatial relationship between endothelial cells and the BrdU$^+$ progenitor cells localized in the vicinity of blood vessels in GCL (Fig. 6c), we performed double-immunolabeling for BrdU and endothelial cell marker von Willebrand Factor (Fig. 6d–f). BrdU$^+$ cells are shown to be localized very close to the microvessels stained by von Willebrand Factor [46, 48]. Some cells are shown to be completely overlapping with von Willebrand Factor$^+$ endothelials cell, while others are not colabeled, but remain in close proximity to endothelial cells (Fig. 6f). These distinct types of BrdU$^+$ cells (Fig. 6f) correspond to endothelial cells or adventitial cells, respectively, as confirmed by immunoelectron microscopy (Fig. 7) [46].

Using immunoelectron microscopy with no counterstaining, BrdU$^+$ nuclei are identified easily at the vascular wall of the CA1 and DG because of their high electron density due to diaminobenzidine. Some of the BrdU$^+$ elongated nuclei are endothelial cells lining the lumen with a characteristic cupped morphology, while others display morphological features of adventitial cells (Fig. 7). These BrdU$^+$ adventitial cells were identified to be either Iba1$^+$ microglia or Down's syndrome cell adhesion molecule (DSCAM)$^+$ neural progenitor cells by immunoelectron microscopy using specific antibodies. The latter are capable of differentiating into immature neurons and/or glial cells in SGZ and GCL [46].

In both the CA1 (Fig. 8, d9) and DG (Fig. 8, d8), microglial encompassment is seen around the endothelial cells, which is more remarkable in the postischemic hippocampus compared to the control. Microglial cells with elongated BrdU$^+$ nuclei can be clearly identified by immunoelectron microscopy (Fig. 8, d9). Because of the slender shape, high electron density and periendothelial localization, the microglial cells are also easily identifiable by conventional electron microscopy (Fig. 8, d8). Neuronal progenitor cells are detected within and outside the vascular basement membrane (Fig. 8, d8), and are considered to be *in vivo* primary precursors of immature DG neurons and astrocytes. Whereas adventitial microglia are seen in both the CA1 and DG, adventitial neuronal progenitor cells are observed only in DG. This appears to be one of the reasons why neurogenesis occurs in DG and not in CA1. Interestingly, after ischemia, the CA1 contained more BrdU$^+$ progenitors than the DG (Fig. 5), but the neuronal progenitors were more abundant in the DG compared to CA1 after ischemia.

The spatial relation between BrdU$^+$ cell cluster containing neural progenitors and Iba1$^+$ microglia in the periadventitial space, is visualized by laser confocal microscopy in an orthogonal projection composed of 4 opti-

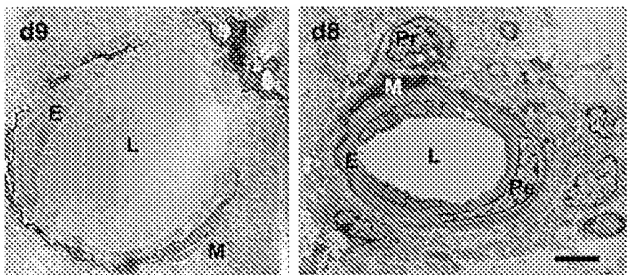

Figure 8. Ultrastructural identification of the adventitial cells. The slender cells encompassing the proliferating capillary lumen (L) are confirmed in both CA1 (d9) and DG (d8). In the former they are positive for microglia (d9: M) marker Iba1 by immunoelectron microscopy, while in the latter they are invested with basement membrane and demonstrate high electron density (d8: M) on conventional electron microscopy. Note that the microglia (M) are distinct from the preexisting pericyte (Pe). The adventitia of DG contains neuronal progenitor cell (Pr). d9: no counterstain, d8: stained with uranyl and lead. Bar, 5 μm.

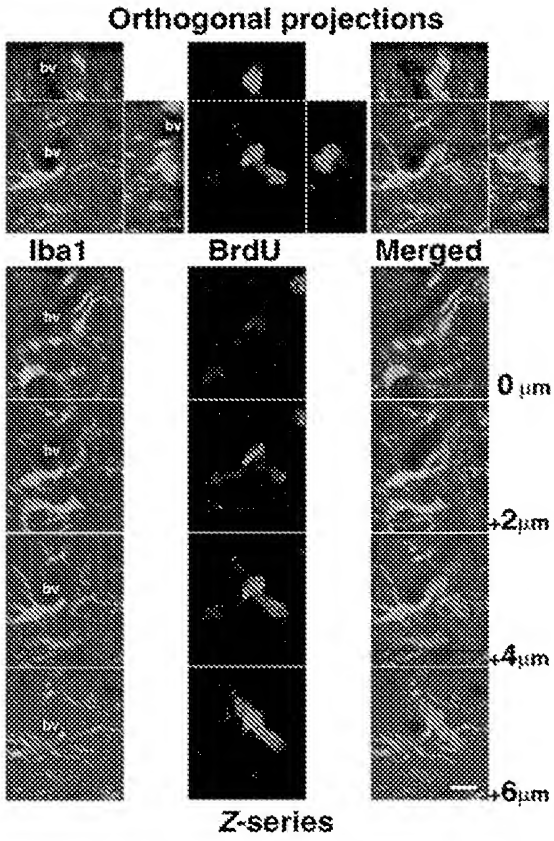

Figure 9. The spatial relation between BrdU$^+$ cell cluster and Iba1$^+$ microglia. An orthogonal projection composing of four optical z-planes (2 μm thick) shows a very close contact between BrdU$^+$ neural progenitor cells (red) and Iba1$^+$ microglia (green) around the proliferating blood vessels (bv). As shown in Fig. 8 (d9), blood vessels appear as an empty space surrounded by Iba1$^+$ microglia (green). Bar, 10 μm.

cal z-planes (2 μm thick) (Fig. 9). BrdU$^+$ neural progenitor cells in the DG tend to be clustered around the microvessels in close promixity to the perivascular Iba$^+$ microglia (Fig. 9). Such BrdU$^+$ cell cluster was formerly shown to encompass newly formed neurons [44]. Consistent with the ultrastructural observations (Fig. 8), these microglial cells encompass the microvessels.

7 DSCAM

Down's syndrome is a major cause of mental retardation and known to be associated with characteristic well-defined brain abnormalities arising after birth, with particular defects in the cortex, cerebellum and hippocampus. The *Dscam* gene maps to 21q22.2q22.3, and encodes the neural cell adhesion molecule DSCAM, which is expressed in the neurons of both the central and peripheral nervous systems, not only during development [59], but also throughout adult life [60]. Recently, Yamashima *et al.* [47] found expression of DSCAM at the SGZ neural progenitor cells. DSCAM and PSA-NCAM double-positive immature neurons and their dendrites are more abundant on day 15, compared to the control (Fig. 10). It is possible that these two cell adhesion molecules play a crucial role for making new synaptic contacts between newly generated and pre-existing neurons.

8 Mechanism of ischemic neuronal death

As shown in Figs. 1–3, Ca^{2+} mobilization, calpain activation, and lysosomal membrane disruption followed by cathepsin release, all occur within the CA1 sector after transient ischemia. Accordingly, in 1998 Yamashima *et al.* [13] formulated the calpain-cathepsin hypothesis as a mechanism of ischemic neuronal death. Although the lysosomal breakdown and translocation of cathepsins and DNase II start from immediately after ischemia, CA1 neuronal death is not complete until day 5. This is presumably because lysosomal leakage becomes maximal on day 3 (Fig. 2) and up-regulation of cathepsin enzyme activity occurs after day 3 [13].

Calpain has been accepted to be involved in various disorders including not only cerebral ischemia but also Parkinson's disease, traumatic brain injury, and myocardial ischemia [61]. Calpain has been demonstrated to be a potential molecular basis also for neuronal degeneration in the Alzheimer's disease brain [62] and in cultured neurons overexpressing amyloid precursor protein [63]. The lysosomal membrane is essentially a physical barrier that prevents hydrolytic enzymes from digesting the cell's constituent proteins, but its disruption and/or permeabilization can cause cell death in pathological states. De Duve and Wattiaux [64] originally suggested such a concept that lethal cell injury occurs as a result of release of hydrolytic enzymes from the disrupted lysosomes. However, unfortunately, this concept did not receive much further attention. Over two decades later, Yamashima *et al.* [13] first proposed a cascade of calpain-induced lysosomal

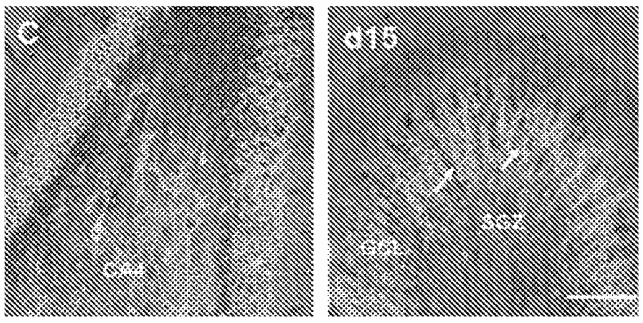

Figure 10. Postischemic up-regulation of DSCAM and PSA-NCAM in the SGZ. DSCAM (green) and PSA-NCAM (red) double-positive newly generated neurons and their dendrites (arrows) remarkably increase on day 15 after ischemia (d15), compared to the control (C). GCL; granule cell layer, Bar, 100 μm

rupture in the primate models of transient brain ischemia. A recent study using *C. elegans* shows that cytoplasmic acidification develops during necrosis [25]. Acidification is required for cell death, because each cathepsin enzymes have an optimal pH. Although most of the cathepsin enzymes function optimally at a low pH within lysosomes, cathepsins B and D can conserve their activity in the acidic cytosol. Artal-Sanz *et al.* [65] used a genetically encoded fluorescent marker to follow lysosome fate during neurodegeneration *in vivo*. Consistent with the data shown in Fig. 2, they reported that, in *C. elegans* neurodegeneration, lysosomes fuse and localize exclusively around the swollen nucleus, with the lysosomal membrane fading. These findings in *C. elegans* concur with those in primates, and, taken together, indicate implication of lysosomal permeabilization and/or rupture for executing cell death.

The question then arises as to what is the mechanism of calpain-induced lysosomal rupture during the postischemic neuronal degeneration? Stabilization of lysosomes by calpain inhibitors or imidazoline drugs, as well as inhibition of cathepsin protease activities, have been shown to rescue CA1 neurons from the ischemic insult. Accordingly, one possibility is that proteolysis of spectrin (fodrin) and its related cytoskeleton by activated calpain may weaken the mechanical strength of the lysosomal membrane. As calpain contributes to cell death by cleaving essential cytoskeletal proteins of neuronal axons [66], it is also possible that calpain acts on microtubules or actins that support lysosomal organelles at an early stage of neurodegeneration. The lysosomal membrane translocation, as shown in Fig. 2, may reflect damage to microtubules or actin motors, which mediate movement of lysosomal organelles. Importantly, most of the known calpain substrates such as signaling molecules, membrane proteins, intracellular enzymes, and structural proteins, have been identified using *in vitro* calpain cleavage assays and do not necessarily reflect target proteins *in vivo*. Identification of intracellular calpain substrates in dying neurons of living animals is needed to precisely understand the mechanism of lysosomal disruption.

Another possibility is that the lysosomal membrane becomes more susceptible to the additional damage exerted by reactive oxygen species (ROS) [67–72]. The incorporation of molecular oxygen into polyunsaturated fatty acids may initiate a chain of cell death-inducing reactions. For example, the ROS, especially hydroxyl radicals, can produce functional alterations in lipids and proteins. It is likely that oxidative lipid damage (lipid peroxidation) contributes to loss of membrane fluidity, and increase of membrane permeability [73]. Notwithstanding the major role played by ROS in cell integrity, the fact that 67–84% of ischemic CA1 neuronal death is inhibited using cathepsin inhibitors [13, 74, 75], implicates calpain-cathepsin cascade rather than oxidative stress as the more critical executer for cell death.

9 Neurogenesis in the SGZ but gliogenesis in the CA1: Why?

Within the SGZ, progenitor cells close to the microvessels are recruited by unknown signals to divide, and their mitogenic recruitment is accompanied by a concurrent angiogenic response [48]. In the postischemic monkeys, precursors in CA1 also show a proliferation in association with a vascular niche [46, 57]. In the CA1, however, we could never confirm generation of new neurons. This suggests that only an angiogenic microenvironment is not sufficient for adult hippocampal neurogenesis, and that other unknown local factors are also required. In gerbils after global ischemia, the majority of $BrdU^+$ cells migrating from SGZ into GCL express certain neuronal markers, whereas those migrating oppositely into the dentate hilus become astrocytes [43]. When normal neural stem/precursor cells are injected into the DG, only those migrating into the SGZ differentiate into neurons, while those remaining outside the SGZ generate only glia [76]. A similar anatomical restriction was found for the multi-potent stem cells transplanted into the subependymal zone and the rostral migratory stream [77–79]. In the CA1, no neurogenesis occurs under normal or pathological conditions, but Nakatomi *et al.* [80] showed *in situ* that activation of endogenous progenitors by intraventricular infusion of FGF-2 and EGF leads to regeneration of CA1 pyramidal neurons after the ischemic brain injury. Certain environmental cues are crucial to direct cell fates of progenitors.

As demonstrated by Louissaint *et al.* [81] in the higher vocal center of adult canaries, to promote maturation and survival of newly generated neurons, an elegant signaling network between angiogenesis and neurogenesis is crucial, including vascular endothelial growth factor (VEGF), brain-derived neurotrophic factor (BDNF), and their receptors. Briefly, subsequent to angiogenic stimulation by VEGF, the endothelial cells secrete BDNF, which presumably stimulates migration of neuronal progenitor cells from the precursor cells and ultimately drives maturation and survival of the newly formed neurons [82]. Such feedback support of young neurons by the endothelial cells is also reported by Leventhal *et al.* [83] in adult rats: the vascular endothelial cells, by secreting BDNF, support outgrowth and survival of newly generated neurons arising from the forebrain subependyma. Both in the postischemic DG and CA1 of monkeys, the immunoreactivity of BDNF is observed within the perikarya of neurons. However, the vascular adventitia, not endothelial cells, only expresses BDNF immunoreactivity in the SGZ and not in the CA1 [46].

At present, we cannot explain exactly why neurogenesis occurs only in the SGZ, whereas CA1 displays merely gliogenesis. One explanation is that the precursor cells in the SGZ of monkeys express pro-neural transcription factors such as Pax6, Emx2 and Ngn2, while precursors in CA1 do not [45]. The distinct expression of not only such

intrinsic factors but also extrinsic factors might be a critical difference. Another important factor contributing to the SGZ/CA1 neurogenic discrepancy is probably DSCAM, which appears closely related to the adult neurogenesis of primates [47]. The differential expression of DSCAM may contribute to the distinct neurogenic potential of precursors in SGZ and CA1. In this sence, the discovery of DSCAM in the neural progenitor cells [47] would provide a novel insight for the regulation of adult neurogenesis as well as understanding pathogenesis of Down's syndrome. Alternative splicing of *Dscam* generates an enormous molecular diversity, with as many as 38 016 different receptors [84–86]. Such large number of structurally unique receptor isoforms would be required to ensure fidelity and precision of neuronal connectivity during progenitor cell migration, synaptic matching and/or axonal extension not only in the fetal brain but also in the adult brain.

10 Role of microglia in the postischemic neurogenesis

In both rodents [43, 87, 88] and monkeys [44, 46, 47], cerebral ischemia can stimulate proliferation and differentiation of neural progenitors in the DG. Remarkable differences between rodents and monkeys are the smaller number of progenitors and the limited extent of neuronal differentiation in the latter [44]. However, similarity in the neurogenic environment exists between the two species. As shown in Fig. 9, after ischemia, SGZ microglial cells are in close contact with $BrdU^+$ progenitor cells during maturation. This is consistent with the finding of Ekdahl *et al.* [89] using rats after the epileptic insult. These findings seem to be important in terms of clarifying the implication of microglia in adult neurogenesis.

Microglia are known to be resident macrophages [90] and immunoeffector cells [91–93] of the brain. They are recognized as "a sensor of the pathological state in the brain" [94]. Microglia are known to have both beneficial and detrimental roles for the brain. For example, in the normal brain, voluntary wheel running leads to a regional increase of newly generated cortical microglia [95]. Such physiological activation of microglia adds a new aspect to the beneficial role of microglial function in the healthy brain. Accordingly, the beneficial effects of microglia might be for the neuronal activity and/or plasticity [96, 97]. In contrast, activated microglia contribute to the ischemic brain injuries either directly via synaptic stripping and neurophagia, or indirectly through the release of cytotoxins [94, 98]. For example, after middle cerebral artery occlusion, stroke-prone spontaneously hypertensive rats sustain more ischemic damage than the normotensive Wistar Kyoto rats. As a causative factor, Marks *et al.* [99] demonstrate a more pronounced microglial response with the inflammatory processes to focal ischemia in the former rats. The release of various cytotoxins from microglia may induce lipid peroxidation, excess release of transmitters and hormones, vascular leakage, edema, necrosis, or changes in ion flow [94, 100, 101].

ELR^+ chemokines and their receptor CXCR2 are not detected in the non-ischemic hippocampus. In contrast, immediately after ischemia, $CD68^+$ microglial cells increase significantly, and express growth-regulated oncogene α and other ELR^+ CXC chemokines. Moreover, $CD68^+$ microglial cells also express the receptor for ELR^+ CXC chemokines [57]. For the adult neurogenesis, microglial activation might be a harmful microenvironment [89] by secreting cytokines such as IL-1β or IL-6, tumor necrosis factor-α, nitric oxide and/or ROS [102–104]. For example, the inhibition of hippocampal neurogenesis after irradiation is accompanied by a marked increase of microglia within the neurogenic zone [105]. In contrast, microglia activation after brain damage may have beneficial effects by promoting release of neurotrophic molecules [106, 107]. Microglia have been shown to produce neurotrophins not only *in vitro* [108–112] but also *in vivo* [109, 113, 114]. Neurotrophins are necessary to support survival and growth of neurons, regulating neuronal function in both the peripheral and central nervous systems [115–119]. In addition to protecting neurons, neurotrophins have been shown to stimulate proliferation of adult-derived neural stem cells and to instruct their differentiation [120–123].

11 Concluding remarks

Two hippocampal sectors, CA1 and DG, show differential vulnerability to cerebral ischemia or neurogenic potential. CA1 is sensitive to transient ischemic injury, while DG is relatively resistant to ischemic insults. CA1 lacks neuronal regenerative capacity, at least in primates, while DG is capable of producing new neurons throughout life. Activation of the calpain-cathepsin cascade due to the ischemic insult contributes to CA1 neuronal necrosis, although apparently not in the DG. Differential expression of intrinsic (transcription factors) or extrinsic (environmental cues) signals in neural precursor cells in the CA1 or DG probably contributes to the discrepancy in neurogenic capacity. Identification of the precise molecular signals governing differential responses of the CA1 and DG neurons or progenitors to ischemic injury will provide new therapeutic targets to prevent or repair the ischemia-injured brain.

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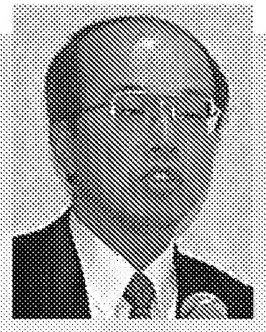

Dr. Yamashima is an academic neurosurgeon and a director of Department of Restorative Neurosurgery, Kanazawa University Graduate School of Medicine, Japan. He is known from his clinical and scientific works about meninges and related disorders, neuronal death and neurogenesis. In addition to hundreds of papers in Japanese, he published another approximately 150 articles in English. Nowadays, he is internationally considered an outstanding researcher in Neuroscience, especially on ischemic neuronal death and adult neurogenesis. Using non-human primates and unique experimental paradigm, he first formulated the „Calpain-Cathepsin hypothesis" as a cause of neuronal necrosis in 1998. Both the lysosomal membrane permeabilization and implications of two cysteine proteases have become widely accepted from studies of *C. elegans* to primates as well as from necrosis to apoptosis. Recently, he discovered the expression of Down's syndrome cell adhesion molecules (DSCAM) in neural progenitor cells of the postischemic hippocampus. In his laboratory, Dr. Yamashima is seeking post-graduate students, foreign research fellows or Post-Doctoral Fellows to develop a strategy for restoring impaired brain functions (http://web.kanazawa-u.ac.jp/%7Emed66/). His hometown Kanazawa is located in the midportion of the west coast of Japan, and is well-known for its marvelous castle, park, universities, fresh seafoods, delicious Sushi and traditional culture. Here one can enjoy fantastic research and beautiful surroundings.

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